ISSN: 0075-5222

The Effects of FGF2 and Ozone Applications to Experimental Hypoxic Ischemic Rat Cerebrum

Leyla BAHAR[1], Mehmet GUL[2], Fatma YILDIZ[3], Bora RESITOGLU[1], Sema ERDEN ERTURK[1], Yalcın CELIK[4]

[1] Vocational School of Health Services, Mersin University, Mersin, Turkey

[2] Department of Hıstology and Embryology, Faculty of Medicine, Inonu Unıversıty, Malatya, Turkey,

[3] School of Health Servıces, Toros Unıversıty, Mersin, Turkey,

[4]Department of Pediatry, Faculty of Medicine, Mersin Unıversıty, Mersin, Turkey

Address for Correspondence Author:

Leyla Bahar, MD, PhD

Vocational School of Health Services

Mersin University, Mersin, Turkey

Tel: +90 324 2347723 Fax: +90 3242346410

leylabahar@mersin.edu.tr

Financial Disclosure: The authors declared that this study has received no financial support.

Running Title: Neuroprotective Effects of FGF2 and Ozone

ISSN: 0075-5222

The Effects of FGF2 and Ozone Applications to Experimental Hypoxic Ischemic Rat Cerebrum

Abstract: Our study aims to show whether the administration of Fibroblast Growth Factor-2 (FGF2) and ozone against neuron-damaging hypoxia-ischemia has any neuroprotective effects in rats exposed to experimental cerebral ischemia-reperfusion. 6 groups were created with each group containing 10 rat that 7d old male Wistar rats. The first group, which was the Sham group, was the one in which a neck dissection was performed while ischemia was not caused. 2nd group, carotid arteries were linked, hypoxia was induced in order to cause hypoxia-ischemia (HI), which is done all groups except sham. The other groups were the ones with 10 µl/ml and 20 µl/ml FGF2 administered. The last groups were the ones with 25 µg/ml and 50 µg/ml ozone administered, routine tissue follow-up procedures were carried out and stained with hematoxylin and eosin stain, and inflammation, eosinophilic cytoplasm, edema in the cerebral cortex, vascular congestion and necrobiotic-necrotic changes were scored and evaluated. In light microscope revealed moderate-to-severe eosinophilic cytoplasm and necrobiotic changes in the HI group when compared to the Sham group and the 20 µl/ml FGF2 group among others. Significant differences in the criteria of inflammation, vascular congestion and edema in the cerebral cortex was detected between the same groups. Kruskal-Wallis test was used for intergroup comparison resulted in a statistically significant difference between all of the study groups (p<

ISSN: 0075-5222

0.05). In our study, morphological recovery was observed depending on the different doses of FGF2 and ozone used to protect the neurons, were fund to be statistically significant.

Keywords: Hypoxic-ischemia, FGF-2, Ozone, The Neuroprotective Effect.

Introduction

Hypoxic-ischemic encephalopathy is an important cause of neurologic dysfunction still seen among the pediatric population. Despite the new imaging techniques for diagnosis and the new approaches for treatment such as whole body/head hypothermia, no significant decrease was observed in the incidence. Studies on incidence and prognosis for the encephalopathies secondary to hypoxia-ischemia representing the most important disease group in the infant and pediatric neurology departments are still important (1,2).

Hypoxia-ischemia in infants are one of the important causes of permanent damage in the central nervous system cells. In hypoxic-ischemic neonatal rats, it was observed that lipid peroxidation increased and/or antioxidant activity decreased (3). As a result of lipid peroxidation in the cell membrane, Oxygen Free Radicals (OFR) were reported to cause damage to DNA and other subcellular organelles, and subsequent cell death. Neonatal brain seems quite vulnerable due to oxidative damage which can be explained by the high concentrations of unsaturated fatty acids, low concentrations of antioxidants, a high level of oxygen consumption, and the presence of redox-active iron (4).

ISSN: 0075-5222

Ischemia plays a crucial role in the hypoxic-ischemic brain injury (HIBI) (5,6). The reperfusion period following hypoxia and ischemia is the phase where the main damage occurs (7). It may be possible to prevent or reduce the damage by employing treatments before the onset of this period. The response of the immature brain to hypoxia and ischemia is different from that of the adult brain. Beside the initial damage by hypoxia, it is known that reperfusion, glutamate and nitric acid neurotoxicity, free radical formation, and calcium accumulation and apoptosis secondary to it, cause irreversible damage in the brain (8). Some researchers carry out studies in order to reveal these mechanisms affecting the brain cells negatively and accordingly take precautions to protect the brain before it is damaged. For this purpose, brain cells are cultured and metabolisms of neurons and glial cells are studied in vitro. Since brain cells consume 20% of the oxygen used by the body, the production and elimination of peroxides are highly important processes. These cells include some defense mechanisms to prevent the cellular accumulation of peroxides and the damage by the peroxide-origin radicals. These mechanisms are the ones with antioxidant effects enabling the destruction of peroxides and protecting against oxidative damage. Cultured brain cells are often used in order to investigate the peroxide metabolism of neural cells. An effective exogenous hydrogen peroxide elimination was achieved for the cultured astrocytes, oligodendrocytes, microglial cells and neurons (9).

While some of the studies aiming at the protection of brain cells deal with the endogenous protection to be provided by hypoxic preconditioning against the subsequent lethal hypoxia, the contents of the relevant mechanism are yet to be

ISSN: 0075-5222

understood. The preconditioning that develops with the recurrent attacks of mild hypoxia increases the resistance of the organism by raising the structural and functional resistance of brain neurons to the subsequent severe hypoxia (10,11). It has been shown that the newborn rat's brain is protected against the subsequent hypoxic ischemic brain damage with the treatment (12,13).

In our study, therapeutic agents to protect the brain cells against hypoxia-ischemia and/or prevent them from being damaged by it were tested. A variety of studies have focused on the effects of ozone and FGF2, whose therapeutic effects have recently been investigated for many diseases, on the neurons in the cerebral cortex tissue. It was reported that oxidative stress caused by ozone exposure induces the repair of brain loss in the hippocampus (14). Low ozone exposure induces the oxidative stress taking part in neurodegenerative diseases. The Forkhead Box O (Foxo) family of the transcription factors regulates the oxidative stress resistance and cell proliferation by activating the oxidative signals. The effects of chronic ozone exposure on Foxo 1a and Foxo 3a activation were investigated in the hippocampus. The results showed that ozone modified the regulatory pathways linked with the antioxidant system and cell cycle, the stimulated neuron inflow, and the apoptotic death (15). Numerous studies have demonstrated that neuronal damage, like the one in Alzheimer's disease, increases the expression and activation of cell cycle regulatory proteins (16). Sun et al. showed that was expressed nestin and growth-associated protein-43 (GAP-43), using exogenous basic fibroblast growth factor (bFGF) in the hippocampus of the brain. Exogenous bFGF may enhance the expression of nestin and GAP-43 in the

ISSN: 0075-5222

brain of neonatal rats with HIBI, which may play a significant role in regeneration of neurons damaged because of hypoxia-ischemia. (17).

Materıals And Methods

The selection of the experimental group and Hypoxic-ischemic creation

In our study, the ethical approval was obtained from the Mersin University Ethics Committee and the study were administered in Experimental Animals Research Laboratory of Mersin University. The Declaration of Helsinki, guide for the care and use of laboratory animals in the relevant field of research. Firstly, groups were determined, as the examples in the literatüre (5). Rats were randomly divided into six equal groups, sham operated control group. 6 groups were formed consisting of 10 Wistar male rats in each group. The first group; Sham group, The carotid arteries of those in group 1 were located, but not were ligated. The rats in this group were not exposed to hypoxia either. The right carotid artery of other group rats, excluding those in the sham group, was tied with 5.0 silk suture. After, these rats were kept in a hypoxia chamber containing 8% oxygen and 92% azote, for two hours. Except sham group, hypoxic ischemia application was made to all groups. The second group; right carotid artery was ligated to create hypoxic ischemia was applied. Then they were taken out and treated with FGF2 and Ozone according to the groups. The other two groups 10µl/ml FGF2 (FGF2-10) and 20µl/ml FGF2 (FGF2-20) were administered via the intraperitoneal route immediately after HIBI was induced. FGF2 (recombinant human FGF basic 146 aa, cat. no. 233-FB-025) solution was prepared. The "Evozone Basic Plus Ozone

ISSN: 0075-5222

Unite" device was used as the ozone generator. In the study, the flow rate of the device is 10ml/sec and the concentration range is 0-8 µg /ml. The last two groups, 25 µg/ml ozone (Ozone-25) and 50 µg/ml of ozone (Ozone-50) were administered via the intraperitoneal route immediately after HIBI was induced.

Light microscopic examination

For the light microscopic examination, the cerebrums of the rats were removed and cut into the small pieces ($1cm^3$). The samples were fixed in 10% buffered formalin, after a series of alcohol and xylene, prepared for routine paraffin embedding. Sections of tissues were cut at 5 µm, mounted on slides, and stained with hematoxylin-eosin (H-E). Slides were examined by a Olimpus BX53 light microscope and Olimpus DS-26 camera and DS Image Analysis System (Olimpus Corporation, Tokyo, Japan). A minimum of 20 fields at 20X magnification were assessed for calculating the mean score of inflamation, edema in cerebral cortex, vascular congestion, necrobiotic-necrotic changes. (maximum total score =12). Light microscopic examination was performed. Histologic Evaluation of “Cerebral cortex and neurons damage between groups” was performed. Light microscopic examination were evaluated in detail by one and the same histologists.

Statistical analysis

Differences between groups were statistically analyzed, "Statistica 6" was held on licensed software. Kruskal-Wallis test after administration, a post-hoc test for differences between groups were performed. 0.05 significance level was taken.

ISSN: 0075-5222

Results

A histologic evaluation of the cerebral cortex and neurons damage between groups was made. Scoring of developing neurodegeneration parameters in the cerebral cortex tissue due to hypoxic-ischemic was performed (Table 1). Graphs were held for the results to be considered statistically significant. In particular, the "inflammation", "eosinophilic cytoplasma" and "Necrotic Necrobiotic Change" terms achieved meaningful results. Histopathological examination of Hematoxylin-Eosin staining in the sham group showed normal morphology. Ischemia group showed many degenerated neurons with karyolitic, karyorectic nucleus necrotic necrobiotic changes and eosinophilic cytoplasma. Comparing the ischemia group with the sham group, there was a significant increase in the number of dead and degenerated neurons in ischemia group ($p<0.05$). When light microscopic examined, especially the sham (Fig. 1a,b) and the other group FGF-20 (Fig. 3b); the results are closer to each other. When compared with sham and the HIBI group (Fig. 2a,b); neurons with necrobiotic changes in moderate to severe levels with numerous eosinophilic cytoplasm were observed in hypoxic ischemia group. Between the same groups; inflammation, vascular congestion and cerebral cortical edema criteria were found and there were significant differences. As shown in Fig.3a and Fig.4a, no statistically significant difference was found when compared with the HIBI group in terms of parameters. In Fig.4b, as seen in Fig.3b; the number of intact neurons was found statistically significant when compared with the HIBI group. Results of statistical analysis by Kruskal-Wallis test were used, a significant difference between the experimental groups was

ISSN: 0075-5222

obtained ($p < 0.05$). As it is shown in graph 1; the Kruskal-Wallis test was applied. There are differences in inflammation in general between groups ($p < 0.05$). There is only this difference between the sham and HIBI ($p<0.05$). In graph 2; in general, "eosinophilic cytoplasma" there are differences in terms of all groups ($p < 0.05$). These differences are in all other groups with the sham ($p<0.05$). In addition, HIBI and FGF-20 are also obvious differences between ($p < 0.05$). Graph 3, it is seen that; "Necrotic Necrobiotic Change" terms, is the difference between all groups in general ($p < 0.05$). Specifically, there are differences among all groups with the sham ($p<0.05$). Also there are between HIBI and FGF-20 and obvious differences between HIBI and Ozone-50 ($p < 0.05$).

Discussion

Perinatal hypoxia-ischemia (HI) is the most common cause of various neurological disabilities with high societal cost in children. Hypoxic-ischemic brain damage is an evolving process, and ample evidence suggests a distinct difference between the immature and mature brain in the pathology and consequences of a brain injury (18). The insufficient blood flow within the cerebrum manifests the clinical symptoms of ischemia; however, the changes are reversible after the restoration of the correct circulation. Five pathways leading to neuron death were determined to be excitotoxicity and ionic imbalance, oxidative stress, inflammation, peri-infract depolarization, and apoptosis (19). Alkan et al. used brain tissue from 7-day old rats to resemble newborn human brain tissue as closely as possible. In their previous studies, they checked the enzyme levels resulting from trauma and investigated the possible protective effect following

ISSN: 0075-5222

oxidative stress. For this purpose, they created hypoxic preconditioning and eventually, they detected increases in superoxide dismutase and (SOD) and gluthathione peroxidase (GPx) levels. Based on the biochemical data, they confirmed the conclusion that oxidative stress increases tolerance to ischemia (3). It is also stated that posthypoxic neurogenesis during neonatal development may be responsible for the protection of the brain (8). Therefore, it is of the utmost importance to better understand the mechanisms underlying the hypoxic-ischemic injury in the neonatal brain to devise effective therapeutic strategies. Effective neuroprotective strategies will include either inhibition of the death effector pathways or induction of their regulatory and survival promoting cellular proteins (18). In neonatal HIBI models, a great variety of pharmacologic agents and methods have been used so far to prevent injury. Being the main purpose of our study as well, discovering new therapeutic methods and agents for the causes of neurodegeneration due to perinatal hypoxia-ischemia for coping with neurodegenerative diseases. FGF2 which is used in our study for this purpose, is a member of the fibroblast growth factor family and encoded by a protein-coding gene. This protein has been implicate on diverse biological processes, such as limb and nervous system development, wound healing, and tumor growth. Since it plays a role in nervous system development, it has been assumed to have neuroprotective effects in our study. When the number of neurons with eosinophilic cytoplasm (Fig. 2a,b), which are formed in the cerebral cortex tissue of the hypoxia-ischemia group and are one of the indicators of neuronal injury, is compared to that of the FGF2-20 group (Fig. 3b), the difference is statistically

ISSN: 0075-5222

significant. Additionally, in the HI group neurons "eosinophilic cytoplasm and necrobiotic necrotic changes" were more common seen, when compared with the FGF2-20 group (Graph 2,3). The decrease in the level of neuronal degeneration in the cerebral cortex tissue of the FGF2 group, which was revealed by the above microscopic examination, is a promising finding that implies the possible presence of neuroprotective effects of FGF2. In their study, Sun et al. observed that the administration of exogenous FGF to the HI-induced neonatal rats increased the levels of Nestin and GAP-43 protein expression. Exogenous FGF may play an important role in the renewal of nerve cells injured due to hypoxia-ischemia. The increased Nestin and GAP-43 protein expression in the hippocampal CA1 region of the neonatal rats may contribute to the neural stem cell activation and post-HI regeneration of the neurocytes. Moreover, treatment with FGF may increase the capability of learning and memory of the neonatal rats (17). Human transgenic FGF1 expression protects the perinatal brain against hypoxia-ischemia damage by taking an effective part in caspase-XIAP signal transmission (20). Perez et al. stated that FGF2 represents an integrator of anxiety behavior and a novel treatment target for the treatment of clinical anxiety and mood disorders (21). Blanco-Alvarez et al. found that the subacute administration of zinc also increases the expression of FGF2 in the early phase after hypoxia-ischemia process and they said that with this way neuroprotective effect in the cerebral HI model (22). Recent studies have further defined the FGF2 cooperates with IL-17 to promote autoimmune infammation (23). Since the neurodegenerative diseases are common and mostly radical treatments could not

ISSN: 0075-5222

found, new molecular targets are searched for in many studies on neuronal injury and neuroprotective effects. Hossain et al. identified the novel neuronal protein NP1 induction in the neonatal brain following HI. And hypoxia-induced NP1 protein induction and neuronal death ($p< 0.001$), demonstrating a specific necessity of NP1 in HI (24). As well as having been investigated the direct neuroprotective effects, recent studies have aimed at revealing the molecular mechanisms of these agents. Thus, translational medical research is carried out on neurodegenerative diseases causing behavioral changes such as neurodegeneration-associated hyperactivity, aggression, cognitive impairment as well as temporal lobe epilepsy (25).

The other agent used by us to prevent or treat the neural injury due to hypoxia-ischemia is ozone. It was reported that ozone injection has effects on locomotor behavior and striatal function, and was said that ozone reduced exploratory behavior but increased freezing behavior (26). Chronic ozone exposure causes increase of reactive oxygen species, which reason an oxidative stress state in the organism. Ozone is one of the main components of photochemical pollution. Considering ozone, the aim of this study was to determine the dose-dependent effect created by ozone in the cerebral cortex and investigate its neuroprotective effects. Another aim was to contribute, at the microscopic level, to the evaluation of the induction of oxidative stress by ozone. Ozone, a pro-oxidant and environmental pollutant, have been noted to have central nervous system effects. Ozone exposure presents a potential model with etiological validity to investigate oxidative stress in depression and antidepressant

ISSN: 0075-5222

action (27). Recently, there have been many studies investigating the effects of ozone not only on nervous system but also dental health, cardiovascular stent treatments, joint pain, testicular torsion, germ elimination. However some researchers, on the other hand stand on new neurotrophic agents against the oxidative stress caused by ozone exposure (28). Swanson et al. stated that while ozone itself can cause health problems, it can also react with chemicals found in a plurality of products to lead to other potentially toxic substances. Despite proponents for its use and the potential practices, toxicity can occur even at environmental levels and may be related to neurologic, respiratory and cardiac incidents (29).

Having concluded that ozone takes an effective part in the nervous system, the study have been revealed that a single subcutaneous injection of ozone reduced the neuropathic pain type behavior and normalized the pro-inflammatory caspases expression (30). Results have showed that a single subcutaneous injection of ozone prevents allodynia and decreases the overexpression of proinflammatory caspase in the orbito-frontal cortices of neuropathic mice (26).

The investigation of the therapeutic effect of two different doses of ozone on neuronal injury in our study revealed that the 25 µg /ml dose did not affect neuronal morphology, whereas the 50 µg /ml dose caused a decrease in the necrobiotic-necrotic changes. Depending on the dose, it has been noted that ozone may be useful on neuron number and morphology. (Fig. 4b), (Graph 3). Zhou et al. investigated the effects of the different doses of ozone on astrocytes. As it is in our work, based on these findings, it was postulated that ozone had dose-

ISSN: 0075-5222

dependent effects and medical ozone therapy reduces oxidative stress (31,32). In a recent study by Khatri et al., they argued that ozone is valuable for therapeutic procedures as an antimicrobial, antihypoxic, analgesic and immunostimulant (33).

The studies on ozone is increasing day by day, resolving mechanisms affecting cells may increase the frequency of use of ozone in our daily lives. Medical applications of ozone therapy are frequently tested. Used in such areas; In removing inner ear damage, in postoperative dental pain, reducing coronary stent restenosis, in spinal pain, enhancing methicillin-resistant Staphylococcus aureus elimination, reducing orthopedic pain.

Consequently, our study has shown that FGF2 and Ozone treatments may be useful for treating damage to the brain in HIBI. By examining brain morphology, it is important that the semiquantitative values are statistically significant. However, there is a need new studies to changes in cell organelles which can not be demonstrated in light microscopy. Transmission electron micrscopy for this, it can be useful to ultra-structurally show neuronal injury parameters and neuroprotective effects.

Acknowledgment

We thank all authors because of the effort during the implementation phase and contribution in our study.

Dısclosure

The authors have no potential conflicts of interest to disclose.

ISSN: 0075-5222

AUTHOR CONTRIBUTION

Study concepts and design: Bahar L, Gül M. Celik Y. Supervision: Bahar L, Gül M, Yıldız F. Resource: Gül M., Resitoglu, B., Celik Y. Data Collection and/or Processing: Erden Ertürk S, Bahar L, Celik Y. Analysis and/or Interpretation: Yıldız F., Erden Ertürk S.; Resitoglu, B. Literature Search - Bahar L. Yıldız F, Erden Ertürk S. Writing: Bahar L, Gül M. Critical Reviews: Bahar L, Gül M.

Approval of final manuscript and agreement of submission: all authors.

References

1. Badr Zahr LK, Purdy I. Brain injury in the infant: the old, the new, and the uncertain. J Perinat Neonatal Nurs 2006; 20:163-75.

2. Kemer S, İATanju, Karademir F, Kul M, C Meral C, Akcan AB, Göçmen İ, Akcan AB. Üçüncü Basamak Bir Sağlık Kuruluşunda Son 3 Yılda İzlenen Hipoksik İskemik Ensefalopatili Olgular ve Kısa Dönem Klinik Seyirleri. J Curr Pediatr 2010; 8:100-4.

3. Alkan T, Gören B, Vatansever E, Sarandöl E. Effects of Hypoxic Preconditioning in Antioxidant Enzyme Activities in HypoxicIschemic Brain Damage in Immature Rats. Turk Neurosurg 2008;18(2):165-71.

4. Buonocore G, Groenendaal F. Anti-oxidant strategies. Seminars in Fetal & Neonatal Medicine. 2007; 12:287-95.

5. Jerome Y.Yager. Animals Models of Hypoxic-Ischemic Brain Damage in the Newborn. Seminars in Pediatric Neurology 2004; 11:31-46.

6. Halliwell B. Oxidative stress and neurodegeneration: where are we now? J Neurochem 2006; 97(6):1634–58.

7. Aşcı S, Demirci S, Aşcı H, Doğuç DK, Onaran İ. Neuroprotective Effects of Pregabalin on Cerebral Ischemia and Reperfusion. Balkan Med J 2016; 33:221-7.

ISSN: 0075-5222

8. Daval JL, Vert P. Apoptosis and neurogenesis after transient hypoxia in the developing rat brain. Semin Perinatol 2004; 28:257-63.

9. Dringen R, Pawlowski PG, Hirrlinger J. Peroxide detoxification by brain cells. Journal of Neuroscience Research 2005;79:157-65.

10. Gidday JM. Cerebral preconditioning and ischaemic tolerance. Nat Rev Neurosci 2006; 7:437-48.

11. Samoilov MO, Lazarevich EV, Semenov DG, Mokrushin AA, Tyul'kova EI, Romanovskii DY, Milyakova EA, Dudkin KN. The adaptive effects of hypoxic preconditioning of brain neurons. Neurosci Behav Physiol 2003; 33:1-11.

12. Jones NM, Bergeron M. Hypoxia-induced ischemic tolerance in neonatal rat brain involves enhanced ERK1/2 signaling. J Neurochem 2004; 89:157-67.

13. Sheldon RA, Aminoff A, Lee CL, Christen S, Ferriero DM. Hypoxic preconditioning reverses protection after neonatal hypoxia-ischemia in glutathione peroxidase transgenic murine brain. Pediatric Research 2007; 61:666-70.

14. Rivas-Arancibia S, Guevara-Guzm´an R., L´opez-Vidal Y., Martinez ER., Zanardo-Gomes M, Angoa-Pérez M, Raisman-Vozari R. Oxidative stress caused by ozone exposure induces loss of brain repair in the hippocampus of adult rats. Toxicol Sci 2010; 113(1):187–97.

15. Gómez-Crisóstomo NP, RodríguezMartínez E, and Rivas-Arancibia S. Oxidative Stress Activates the Transcription Factors FoxO 1a and FoxO 3a in the Hippocampus of Rats Exposed toLow Doses of Ozone. Oxidative Medicine and Cellular Longevity 2014;Article ID 805764, 1-8.

16. Hernandez-Ortega K, Arias C. ERK activation and expression of neuronal cell cycle markers in the hippocampus after entorhinal cortex lesion. J Neurosci Res 2012; 90(11):2116–26.

17. Sun JQ, Cao YT, Liu HQ, Deng WA. Neuroprotective effects of exogenous basic fibroblast growth factor on the hypoxic-ischemic brain damage of neonatal rats. Zhonghua Er Ke Za Zhi. 2007; 45(5):354-59.

18. Hossain MA. Hypoxic-ischemic injury in neonatal brain: involvement of a novel neuronal molecule in neuronal cell death and potential target for neuroprotection. Int J Dev Neurosci 2008; 26(1):93-101.

ISSN: 0075-5222

19. Bielewicz J, J Kurzepa, M Łagowska-Lenard, H Bartosik-Psujek. The novel views on the patomechanism of ischemic stroke. Wiad Lek 2010; 63:213-20.

20. Russell JC, Szuflita N, Khatri R, Laterra J, Hossain MA. Transgenic expression of human FGF-1 protects against hypoxic-ischemic injury in perinatal brain by intervening at caspase-XIAP signaling cascades. Neurobiol Dis 2006; 22:677–90.

21. Perez JA, Clinton SM, Turner CA, Watson SJ, Akil H. A New Role for FGF2 as an Endogenous Inhibitor of Anxiety**.** J Neurosci 2009; 29(19): 6379–87.

22. Blanco-Alvarez V. M, Soto-Rodriguez G, Gonzalez-Barrios JA, Martinez-Fong D, Brambila E, Torres-Soto M, Aguilar-Peralta K, Gonzalez-Vazquez A, Tomás-Sanchez C, Limón D, Eguibar JR, Ugarte A, Hernandez-Castillo J, Leon-Chavez BA. Prophylactic Subacute Administration of Zinc Increases CCL2, CCR2, FGF2, and IGF-1 Expression and Prevents the Long-Term Memory Loss in a Rat Model of Cerebral Hypoxia-Ischemia. Neural Plast. 2015; 2015: 375391.

23. Shao X, Chen S, Yang D, Cao M, Yao Y, Wu Z, Li N, Shen N, Li X, Song X, Qian Y. FGF2 cooperates with IL-17 to promote autoimmune inflammation. Scientific Reports. 2017; 7:7024. doi:10.1038/s41598-017-07597-8.

24. Hossain MA, Russell JC, O'Brien R, Laterra J. Neuronal pentraxin 1: a novel mediator of hypoxicischemic injury in neonatal brain. J Neurosci 2004; 24:4187–96.

25. Corvino V, Marchese E, Michetti F, Geloso MCNeuroprotective strategies in hippocampal neurodegeneration induced by the neurotoxicant trimethyltin. Neurochem Res 2013; 38(2):240-53.

26. Rivas-Arancibia S, Dorado-Martínez C, Colin-Barenque L, Kendrick KM, de la Riva C, Guevara-Guzmán REffect of acute ozone exposure on locomotor behavior and striatal function. Pharmacol Biochem Behav 2003;74(4):891-900.

27. Mokoena ML, Harvey BH, Oliver DW, Brink CB. Ozone modulates the effects of imipramine on immobility in the forced swim test, and nonspecific parameters of hippocampal oxidative stress in the rat. Metab Brain Dis 2010;25(2):125-33.

28. Pinto-Almazán R, Rivas-Arancibia S, Farfán-García ED, Rodríguez-Martínez E, Guerra-Araiza C Neuroprotective effects of tibolone against oxidative stress induced by ozone exposure. Rev Neurol 2014;58(10):441-8.

29. Swanson T, Chapman J. Toxicity Ozone. StatPearls. Treasure Island (FL): StatPearls Publishing; 2017.

30. Fuccio C, Luongo C, Capodanno P, Giordano C, Scafuro MA, Siniscalco D, Lettieri B, Rossi F, Maione S, Berrino L. A single subcutaneous injection of ozone prevents allodynia and decreases the over-expression of pro-inflammatory caspases in the orbito-frontal cortex of neuropathic mice. Eur J Pharmacol 2009;603(1-3):42-9.

31. Zhou NB, Fu ZJ, Sun T. Effects of different concentrations of oxygen-ozone on rats' astrocytes in vitro. Neurosci Lett 2008;441(2):178-82.

32. Tusat M, Mentese A, Demir S, Alver A, Imamoglu M. Medical ozone therapy reduces oxidative stress and testicular damage in an experimental model of testicular torsion in rats. Int Braz J Urol 2017 Jun; 14:43. doi:10.1590/S1677-5538.IBJU.2016.0546.

33. Khatri I, Moger G, Kumar NA. Evaluation of effect of topical ozone therapy on salivary Candidal carriage in oral candidiasis. Indian J Dent Res 2015; 26(2):158-62.

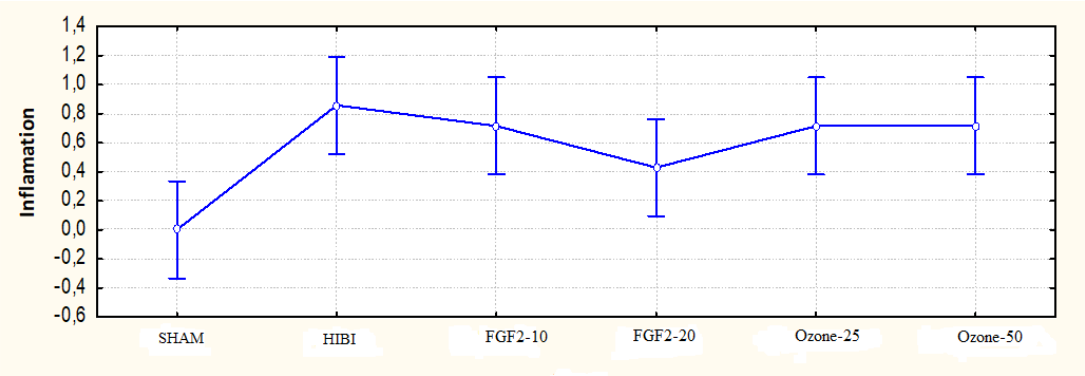
Table And Graphs

Table 1: Scoring of developing neurodegeneration parameters in the cerebral cortex tissue due to hypoxic-ischemic (Inflamation, Edema, Vascular congestion and Necrobiotic-necrotic Changes)

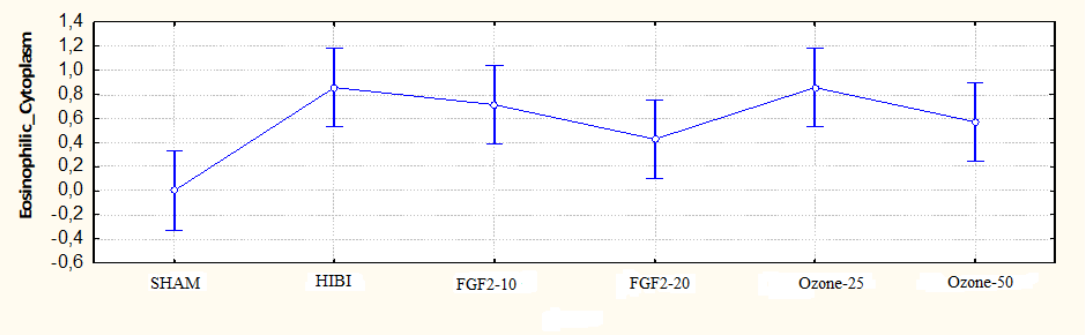
Score	Inflamation	Edema in Cerebral Cortex	Vascular Congestion	Necrobiotic-necrotic Changes
0	Absent	Absent	Absent	Absent
1	Present	mild	mild	mild-moderate nekrobiyoz
2	-	moderate	moderate	evident nekrobiyoz
3	-	severe	severe	mild-moderate necrosis
4	-		Absent	evident necrosis

ISSN: 0075-5222

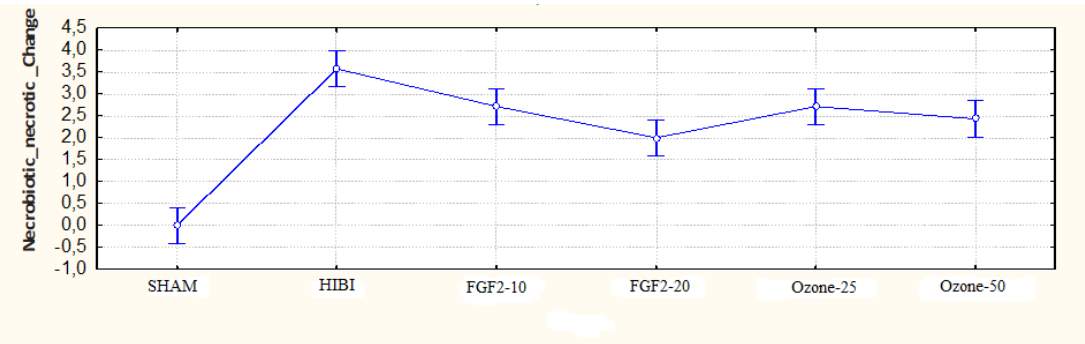
Graph 1.



Graph 2.



Graph 3.



ISSN: 0075-5222

Figures

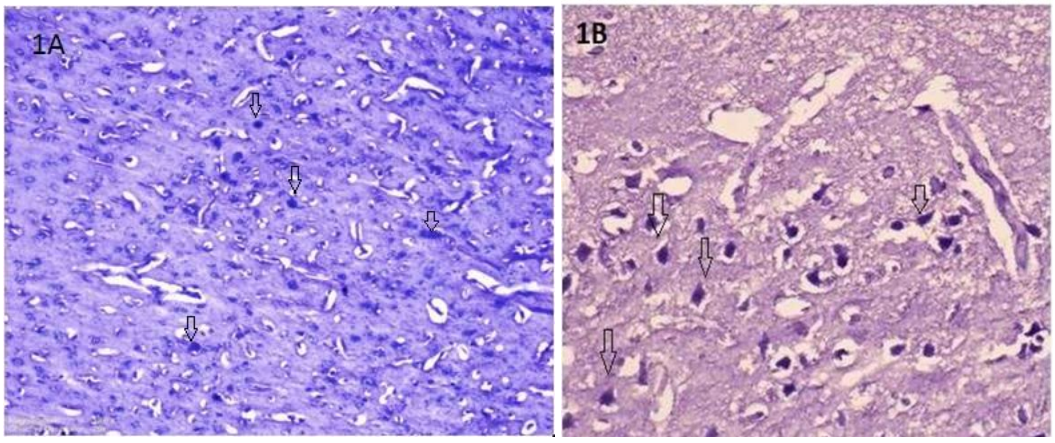


Fig.1. a-b. Sham group, which can be seen above, watched the newborn rat normally brain tissue sections, arrow; normal neurons. Hematoxylin and Eosin (H-E) staining X20,X40.

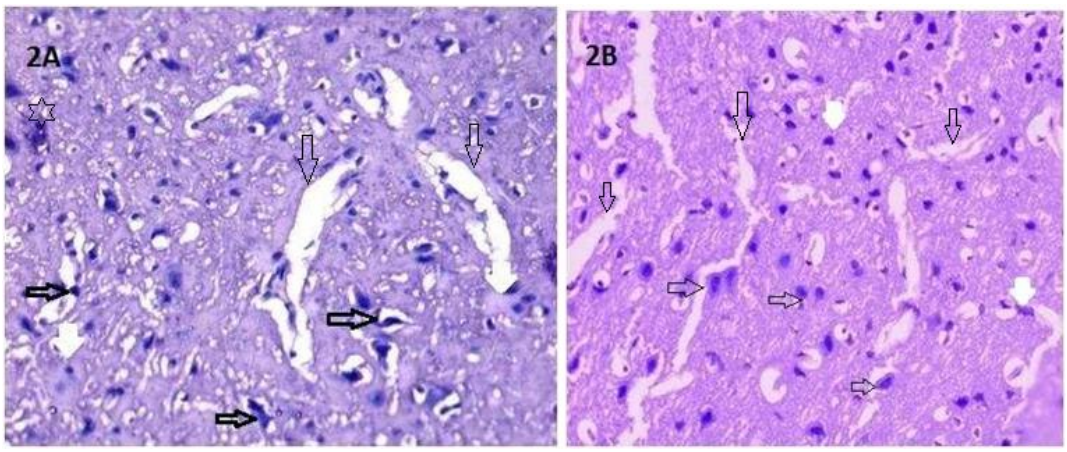


Fig.2. a-b. In the group treated with hypoxic, which can be seen above brain tissue; a large amount of eosinophilic cytoplasm and marked neurodegeneration being monitored; right arrow; with eosinophilic cytoplasm neuron, white arrow-head; neurodegeneration, down arrow; edematous areas. H-E staining X40.

ISSN: 0075-5222

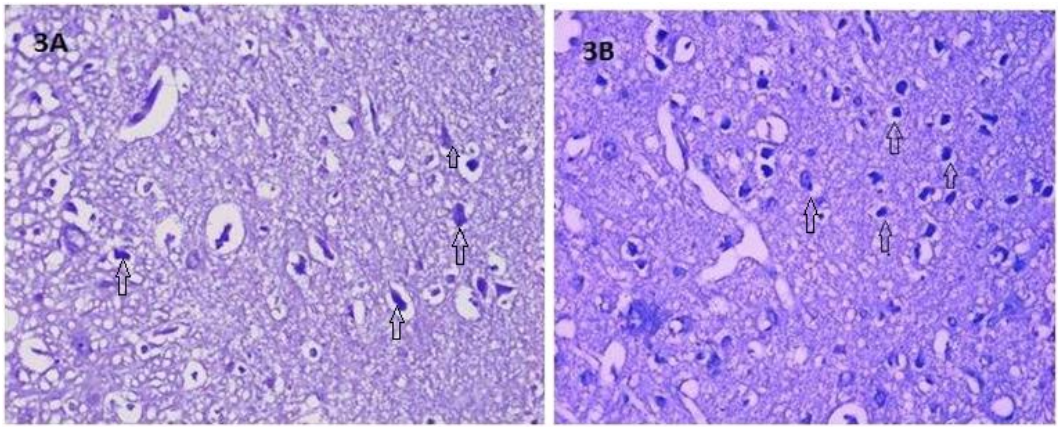


Fig.3. a-b. FGF2, 10 and 20 µl/ml was carried out in two separate groups of brain neurons and monitored neurodegenerative symptoms fewer neuron and glial cells. Up arrow; neurons, H-E, X40.

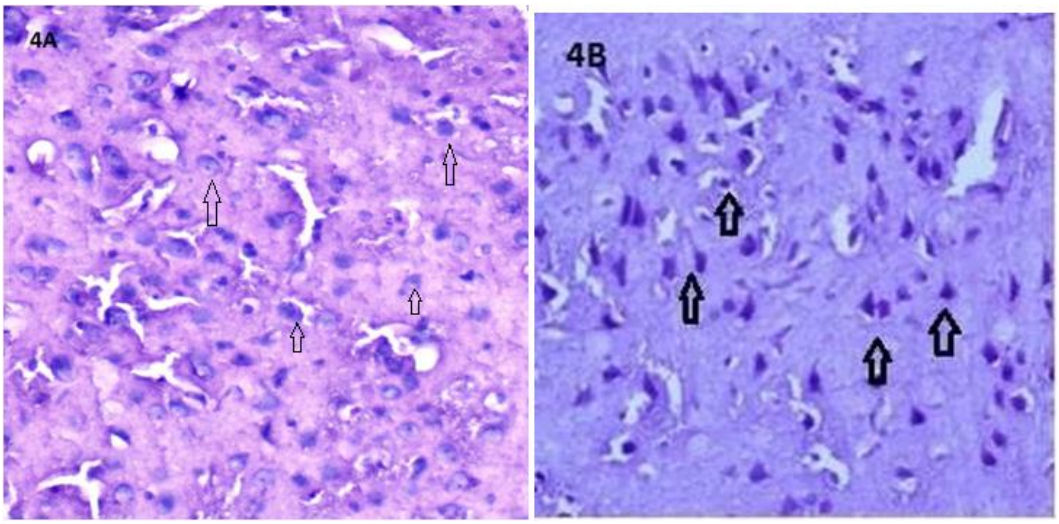


Fig.4. a-b. Ozone 25 µg /ml and 50 µg /ml brain tissue of group administered. Up arrow; normal neurons
Histologic Evaluation of Cerebral Cortex and Neurons Damage Between Groups. H-E staining, X40.